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Glucose enzyme electrode using cytochrome b_{562} as an electron mediator

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Abstract

We demonstrate the construction of glucose sensors employing pyrroloquinoline quinone (PQQ) glucose dehydrogenase (PQQGDH) from *Acinetobacter calcoaceticus* and glucose oxidase (GOD) from *Aspergillus nigar* coupled with *Escherichia coli* soluble cytochrome b_{562} (cyt b_{562}) as electron acceptor. PQQGDH and GOD do not show direct electrochemical recycling of the prosthetic group at the electrode surface leading to a corresponding current signal. We constructed PQQGDH and GOD electrodes co-immobilized with 100-fold molar excess of cyt b_{562} and investigated the electrochemical properties without synthetic electron mediators. PQQGDH/cyt b_{562} and GOD/cyt b_{562} electrodes both responded well to glucose whereas no current increase was observed from the electrode immobilizing enzyme alone. The detection limits for the PQQGDH/cyt b_{562} and GOD/cyt b_{562} electrodes were 0.1 and 0.8 mM, respectively, and their linearity extended to over 2 and 9 mM, respectively. These results demonstrate that a sensor system can be constructed without a synthetic electron mediator by using a natural electron acceptor. Furthermore, we have demonstrated the potential application of cyt b_{562} in direct electron transfer type sensor systems with oxidoreductases whose quaternary structure do not contain any electron transfer subunit.

Keywords: Glucose enzyme sensor; Electron transfer; *Escherichia coli* cytochrome b_{562}; PQQ glucose dehydrogenase; Glucose oxidase

1. Introduction

Glucose oxidase (GOD) is the most popular sensor constituent of blood glucose monitoring because of its high substrate specificity for glucose and stability. However, due to its inherent problem that GOD utilizes oxygen as the electron acceptor, glucose dehydrogenase (GDH) possessing pyrroloquinoline quinone (PQQ) prosthetic group is being considered as the ideal glucose sensor enzyme.

In most oxidoreductases, including GOD and PQQGDH, the prosthetic group is buried deeply within the protein shell. Direct electrochemical recycling of the enzyme's prosthetic group at the electrode surface leading to a corresponding current signal is, therefore, rarely encountered. To solve this, artificial electron mediators are often used in oxidoreductase-based biosensors. Several artificial electron mediators have been reported, such as phenazine methosulphate (PMS), potassium ferricyanide, $K_4[Fe(CN)_6]$, quinones, and Os-complexes. Considering the currently increasing interest in the development of implantable enzyme sensors, such as the continuous glucose monitoring system, mediatorless or direct electron transfer enzyme sensors are preferable, compared with those utilizing toxic low molecular weight artificial electron mediators.

Multicofactor enzymes, most consisting of more than one subunit, were recently shown to display direct electron transfer with the electrode. These include enzymes containing both PQQ and heme, such as D-fructose dehydrogenase (Khan et al., 1991; Ikeda et al., 1991) and alcohol dehydrogenase (QH-ADH; Ramanavicius et al., 1999), as well as enzymes containing both

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FAD and heme, such as *p*-cresolmethylhydroxylase, fumerate reductase (Turner et al., 1999), flavocytochrome c_{552} (Guo et al., 1990), D-gluconate dehydrogenase (Darder et al., 2000), and cellobiose dehydrogenase (Larsson et al., 2000; Lindgren et al., 2000). The polypyrrole entrapped within QH-ADH able to directly transfer electrons via the conducting polymer to the electrode surface. The electron transfer properties of this multicofactor enzyme adsorbed and covalently bound to self-assembled thiol monolayers and bare electrode surfaces have been investigated more closely (Ramanavicius et al., 1999). While the dissolved enzyme is able to transfer electrons to the electrode via heme *c* as well as via the more deeply buried PQQ (first adsorption–chemical reaction–desorption mechanism), a specific orientation could be observed for the adsorbed QH-ADH on hydrophobic electrode surface, as well as for adsorbed and covalently bound QH-ADH on negatively charged thiol monolayers. In these cases, the heme *c* units are pointing towards the electrode surfaces resulting in an optimized direct electron transfer rate.

These experimental evidences encouraged us to develop an artificial electron transfer subunit based on heme containing proteins, such as cytochromes, for the oxidoreductase whose quaternary structure does not contain any electron transfer subunit, such as GOD and PQQGDH. We focused on the application of *Escherichia coli* cytochrome b_{562} (cyt b_{562}). Cyt b_{562} is a 12.7 kDa monomeric protein (Itagaki and Hager, 1966) localized in the periplasm of *E. coli* (Nikkila et al., 1991), whose physiological function remains unknown. The protein adopts a four-helical bundle fold and coordinates to the heme iron through ligands provided by methionine 7 and histidine 102, residues located close to the termini of the polypeptide. It is assumed to be a simple electron carrier shuttling electron between other (membrane bound) redox centers, but no natural partners have yet been identified. The ease of overproduction in *E. coli* cyt b_{562} (Nikkila et al., 1991; Barker et al., 1995) and availability of high resolution structure of both holo- and apo-proteins (Hamada et al., 1995; Feng et al., 1994) make this cytochrome an excellent starting point for the generation and study of cytochromes with novel redox and electron transfer properties.

We recently reported that the co-immobilization of PQQGDH and cyt b_{562} significantly facilitates the artificial electron mediator-based glucose enzyme sensor current response (Okuda et al., 2002). We demonstrate in this paper that an electron transfer protein, cyt b_{562}, can be utilized as the electron mediator to construct a glucose sensor system. This achievement of the utilization of a protein as the electron mediator in an enzyme sensor system has never been reported.

2. Material and method

2.1. Materials

GOD from *Aspergillus nigar* and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louise, USA). Recombinant PQQGDH (water soluble PQQGDH; PQQGDH-B or s-GDH) was prepared using *E. coli* DH5α as the host strain as described previously (Sode et al., 1994, 2000; Okuda et al., 2002). *E. coli* DH5α was transformed with the pTrc99A expression vector (Pharmacia, Sweden) containing a PQQGDH structural gene and cultivated as described previously (Sode et al., 1994), except for the addition of 1 mM $CaCl_2$ instead of $MgCl_2$. The cells were harvested after the late log phase, resuspended in 10 mM phosphate buffer pH 7.0 containing 5 mM $MgCl_2$, and disrupted by French pressure cell (110 MPa). The Sample was then subjected to ultracentrifugation ($160\,500 \times g$, 1.5 h, 4 °C), followed by dialysis in 10 mM potassium phosphate buffer pH 7.0. The resulting supernatant was applied to a CM-Toyopearl 650 M cation exchange column (TOHSOH, Japan) equilibrated with 10 mM potassium phosphate buffer pH 7.0 and washed with two column volumes of the same buffer. The enzyme was eluted with a linear gradient of 0–0.2 M NaCl in 10 mM potassium phosphate buffer, pH 7.0. The purified enzyme, found to be electrophoretically homogeneous by silver staining on SDS-PAGE, was utilized for kinetic studies. GOD, PQQGDH-B, and cyto b_{562} were all wild type.

2.2. Preparation of holo- and apo-cyt b_{562}

Recombinant cyt b_{562} was produced in *E. coli* strain DH5α harboring pTrc-cybC, the pTrc99A expression vector containing the cyt b_{562} structural gene, cultured in L medium at 37 °C. Upon reaching $OD_{600} = 1$, the cells were induced with 300 μM IPTG and the incubation was continued for 18 h. The cells were harvested by centrifugation and periplasmic fraction was obtained by freeze-thaw lysis using 10 mM MOPS buffer, pH7.2. The material was absorbed onto DEAE-5PW column (TOHSOH) and eluted with 200 mM NaCl in the same buffer. Eluted protein was separated by a size exclusion column (Hiload Column, Pharmacia), and the purified protein was evaluated by SDS-PAGE. Recombinant cyt b_{562} was expressed in the holo-form and apo-cyt b_{562} was prepared as described by Feng and Sligar (1991).

2.3. Preparation of PQQGDH/cyt b_{562} and GOD/cyt b_{562} co-immobilized electrode

MOPS buffer (10 mM, pH 7.0) containing 25U (0.67×10^{-10} mol) of PQQGDH or 5U GOD (3.3×10^{-10} mol) and 100-fold molar excess of cyt b_{562} or

the same mass of BSA instead of cyt b_{562} was mixed with carbon paste (0.5 g graphite powder mixed with 0.3 ml paraffin liquid) and lyophilized. The enzyme/cyt b_{562} a ratio was optimized according to previous observations (Okuda et al., 2002). We previously demonstrated that an excess molar ratio of cyt b_{562} to PQQGDH is essential for efficient electron transfer between enzyme and cyt b_{562}. In this study we compared the sensor responses with different ratios ranging from 10- to 200-fold molar excess of cyt b_{562}. Our results show an increase in sensor signal with increasing molar excess of cyt b_{562}, reaching a maximum at around 100-fold molar excess. We therefore, used an enzyme: cyt b_{562} molar ratio of 1:100.

The lyophilized mixture was then packed into the end of a carbon electrode (3 mm in diameter, BAS Inc, West Lafayette, USA) and fixed with 1% glutaraldehyde solution for 30 min and washed with 10 mM Tris–HCl buffer (pH 7.0). The electrode was then allowed to undergo holo-formation in 10 mM MOPS buffer (pH 7.0) containing 5 μM PQQ and 1 mM $CaCl_2$ at 4 °C for at least 30 min, washed with 10 mM MOPS (pH 7.0), and stored at 4 °C until use. PQQGDH/cytochrome co-immobilized carbon paste electrode was immersed in 10 mM MOPS buffer, pH 7.0.

2.4. Electrochemical measurement

An Ag/AgCl electrode (Model RE-1, BAS Inc.) and a Pt wire were used as reference and counter electrodes, respectively. The enzyme electrode (3 mm diameter, BAS Inc.), reference electrode, and counter electrode were joined to a 10-ml water-jacket cell (BAS Inc. Model VC-2) through holes in its Teflon cover. The potential was controlled by a potentiostat HA151 (HOKUTO-DENKO, Tokyo, Japan) in a three-electrode cell and currents were recorded with a chart recorder (Ohkura electric company, Tokyo, Japan). All measurements were carried out at 25 °C in 10 ml of buffer with magnetic stirring (250 rpm). PQQGDH was in 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ for stabilization of the holo form and GOD was in 100 mM potassium phosphate buffer (pH 7.0). The physiologically relevant pH 7 was chosen for both systems. The applied potentials for the calibration curve measurements of PQQGDH and GOD electrodes were +200 and +250 mV versus Ag/AgCl, respectively. The applied potentials were chosen considering the redox potential of +189 mV for cyt b_{562} versus SHE (Barker et al., 1996) and preliminary tests that showed no significant difference with 200, 220 and 250 mV. The calibration curve measurements were carried out with consecutive 100-μl injections of glucose solution into the reaction cell. Cyclic voltamogram (CV) measurements of current response to glucose were carried out using the same electrode in the absence of electron mediator. The potential was cycled between −300 and +300 mV with a sweep rate of 50 mV s^{-1}.

3. Result

3.1. PQQGDH/cyt b_{562} electrode

Fig. 1 shows the CVs of the electrodes immobilizing PQQGDH, PQQGDH with BSA, or PQQGDH with cyt b_{562}. The PQQGDH and PQQGDH/BSA electrodes did not show any redox current. In contrast, a redox current was clearly observed with the electrode immobilizing both PQQGDH and cyt b_{562}. BSA was used in control experiments to confirm that the observed redox current with the PQQGDH/cyt b_{562} electrode did not result from a stabilization effect by the excess protein during electrode preparation. These results indicate that PQQGDH itself did not have the ability to transfer electron directly to electrode through its PQQ redox center. Moreover, the current observed with the PQQGDH/cyt b_{562} electrode increased with the addition of glucose in the reaction vessel. The electrode immobiiizing cyt b_{562} alone showed a CV curve similar to that for the PQQGDH/cyt b_{562} electrode (data not shown), indicating that the CV curve was derived from cyt b_{562}.

We then investigated the correlation between current increase and glucose concentration in the solution (Fig. 2) on the enzyme electrode immobilizing both PQQGDH and cyt b_{562}. The observed current increase was proportional to glucose concentration, with a minimum detectable concentration of 0.1 mM and the linearity extending to 2 mM. The sensitivity of the sensor was 43.2 μA M^{-1} cm^{-2}. Comparing the sensitivities of 3 different electrodes prepared with the

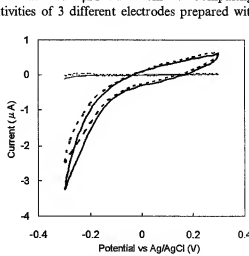

Fig. 1. CVs of the electrodes immobilizing PQQGDH (thin line), PQQGDH with BSA (dotted line), or PQQGDH with cyt b_{562} with (thick line) or without (thick dashed line) 20 mM glucose. The currents were measured in 10 mM MOPS buffer (pH 7.0) containing 1 mM $CaCl_2$ at 25 °C. The sweep rate was 50 mV s^{-1}. The PQQGDH: cyt b_{562} molar ratio was 1:100.

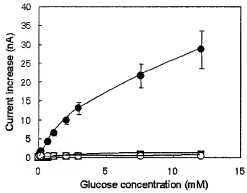

Fig. 2. Calibration curves of PQQGDH-B/cyt b_{562} electrode. GDH was co-immobilized on the electrode with a 100-fold molar excess of either holo cyt b_{562} (closed circles) or apo-cyt b_{562} (closed squares), or immobilized alone (open circles). Currents were measured in 10 mM MOPS (pH 7.0) containing 1 mM $CaCl_2$ at 25 °C with a potential of +200 mV vs. Ag/AgCl. Error bars show the standard deviation ($n = 3$, 14.9±1.8 nA at 12 mM). Sensitivity was 43.2 μA M^{-1} cm^{-2} (linear range; 0.1–2.0 mM, $R^2 = 0.9583$).

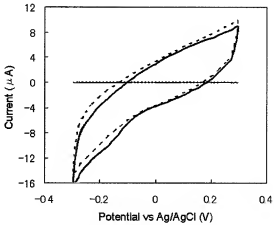

Fig. 3. CVs of the electrodes immobilizing GOD (thin line), GOD with BSA (dotted line) or GOD with cyt b_{562} with (thick line) or without (dashed line) glucose. The currents were measured in 100 mM potassium phosphate buffer (pH 7.0) at 25 °C. The sweep rate was 50 mV s^{-1}. The GOD: cyt b_{562} molar ratio was 1:100.

same components showed less than 15% error, thus confirming the reproducibility of the sensor signals.

However, no current increase was observed from the electrode immobilizing PQQGDH alone, even at glucose concentration higher than 10 mM. In order to confirm the role of cyt b_{562} in this electrode response, we prepared apo-cyt b_{562} in which heme was extracted prior to preparation of the enzyme electrode and then co-immobilized with PQQGDH. The electrode immobilizing both PQQGDH and apo-cyt b_{562} did not show a glucose-dependent signal increase. Apo-cyt b_{562} was used as a control for the same reason as BSA, to ensure that the results do not reflect a protein stabilization effect during the lyophilization and immobilization of PQQGDH. These results confirm the electron transfer role of cyt b_{562} in the electrode system. By co-immobilizing PQQGDH with cyt b_{562}, the electrode can function as a glucose enzyme sensor that does not require synthetic electron mediators and PQQGDH can transfer electrons from its PQQ redox center to the electrode via cyt b_{562}.

3.2. GOD/cyt b_{562} electrode

Similar to the investigation of the PQQGDH-based electrodes, GOD-cyt b_{562} co-immobilized electrodes were investigated for their ability to transfer electrons. The CV was investigated for the electrode immobilizing GOD alone or together with either cyt b_{562} or BSA (Fig. 3). As in the PQQGDH-based electrodes, no redox current was observed from the electrodes immobilizing either GOD alone or GOD and BSA, indicating that GOD itself does not have the ability to transfer electrons directly from its FAD redox center to the electrode. In contrast, the co-immobilized GOD/cyt b_{562} electrode clearly showed a redox current, which increased with the addition of glucose in the solution.

We then investigated the correlation between sensor response and glucose concentration in the reaction vessel (Fig. 4). With the increase of glucose concentration, the anodic current increased for the GOD/cyt b_{562} electrode, whereas no current increase was observed from the electrode immobilizing only GOD or GOD/BSA. The detection limit was 0.8 mM with the linearity extended to 20 mM and a sensitivity of 12.2 μA M^{-1}. The sensor signals of three different electrodes prepared with the same components showed less than 9% error, again confirming the reproducibility.

Therefore, cyt b_{562} also functioned as an electron mediator between GOD and electrode, thus achieving a GOD-based glucose enzyme sensor without additional synthetic electron mediator.

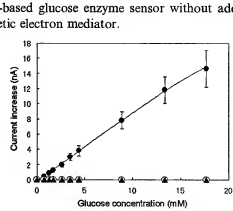

Fig. 4. Calibration curves of GOD/cyt b_{562} electrode. GOD was co-immobilized on the electrode with either a 100-fold molar excess of cyt b_{562} (closed circles), or an equivalent mass of BSA (open circle), or immobilized alone (closed triangle). Currents were measured in 100 mM potassium phosphate buffer (pH7.0) at 25 °C with a potential of +250 mV vs. Ag/AgCl. Error bars show the standard deviation ($n = 3$, 11.85±2.84 nA at 17.7 mM). Sensitivity was 12.2 μA M^{-1} cm^{-2} (linear range: 0.8–9.0 mM, $R^2 = 0.9954$).

4. Discussion

In this paper, we demonstrated that cyt b_{562} can be utilized as an artificial electron transfer subunit for an oxidoreductase that, as GOD and PQQGDH, does not have an electron transfer subunit within its quaternary structure. This is the first report of an enzyme-based sensor consisting of an oxidoreductase coupled to an electron transport protein that is not its natural partner in vivo. Fig. 5 shows the schematic diagram of the proposed electron transfer between enzyme and electrode via cyt b_{562}. GOD has FAD as its redox center, whereas PQQGDH has PQQ as its redox center. The versatility of cyt b_{562} application was demonstrated in this study, as both GOD/cyt b_{562} and PQQGDH/cyt b_{562} electrodes achieved electron transfer in the absence of synthetic electron mediators. This system can be applied to a variety of oxidoreductases that do not have electron transfer subunits to construct enzyme sensors without synthetic electron mediators. The improved current was observed with both electrodes (Figs. 2 and 4). The sensor responses of PQQGDH/cyt b_{562} and GOD/cyt b_{562} electrodes maintained linearity up to 20 and 9 mM, respectively, which is comparable with sensors using synthetic chemical mediator (PQQGDH: 1 mM) (Takahashi et al., 2000) and GOD: 20 mM (Guerrieri et al., 1998).

The stability of an enzyme sensor depends on the stability of the proteins employed in the sensor system. PQQGDH is a relatively unstable enzyme, with a half-life of less than 10 min at 55 °C (Sode et al., 2000). Cyt b_{562} is a stable protein with its Tm reported to be 67.2 °C (Feng and Sligar, 1991). The stability of a glucose sensor employing PQQGDH and cyt b_{562} may, therefore, depend on the stability of PQQGDH, whereas, a sensor employing GOD and cyt b_{562} may depend on the stability of cyt b_{562}, considering the extremely high stability of GOD.

We previously reported that an excess molar ratio of cytochrome c molecule to PQQGDH is essential for achieving facilitated electron transfer in the enzyme sensor employing synthetic electron mediator (Okuda et al., 2002). Because both GDH and GOD are not physiological partners of cyt b_{562}, electron transfer rates might not be optimal. We therefore, utilized a 100-fold molar excess of cyt b_{562} to PQQGDH and GOD. Further development for the ideal artificial electron transport proteins for PQQGDH and GOD may require improvement of the affinity and orientation between cyt b_{562} towards these redox enzymes.

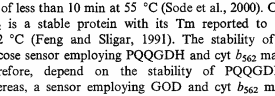

Fig. 5. Schematic diagram of the proposed electron transfer between enzyme and electrode via cyt b_{562}.

The novelty of this study is the utilization of a protein as electron mediator, which has two major advantages. Firstly, the application a protein as the electron mediator will facilitate future construction of the direct electron transfer type enzyme sensor. Our achievement in the application of electron transfer protein as the electron mediator for the sensor system encourages us to engineer the protein to be an 'artificial electron transfer subunit' that has high affinity towards the enzyme. Although an excess amount of cyt b_{562} is essential in the current status, further protein engineering approaches may eliminate this need. Approaches using hemoprotein as an electron mediator have been reported; for example, an enzyme electrode employing cellobiose dehydrogenase (CDH) using cyt c (Fridman et al., 2000; Gilardi et al., 2001) and nitrite reductase (NR) using a synthetic four-helix bundle hemoprotein (Katz et al., 1998). However, both CDH and NR have heme in their electron transfer part. In contrast, our study demonstrated the potential application of a natural electron acceptor as electron transfer subunit for the oxidoreductase that does not possess an electron transfer part.

The second advantage of using a protein as electron mediator is its low toxicity level. Several reagentless GOD-based sensors have been reported. Although they showed high current density and adequate detection limit and linear range, these reagentless enzyme sensors were constructed employing artificial organic electron mediators, such as ferrocene (Gun and Lev, 1996) or Os redox polymer (Ohara et al., 1993). Such organometallic compounds could not be utilized in vivo due to their potential cytotoxicity. On the other hand, as the electron mediator we used a protein, cyt b_{562}, which is a relatively harmless substance.

Our objective was to demonstrate the potential application of cyt b_{562} as an electron acceptor for PQQGDH and GOD, and as an electron mediator between these enzymes and electrode. We therefore, employed the sensor system in experimental conditions in which we observed the electron facilitating ability of cyt b_{562} (Okuda et al., 2002). For the practical application of this sensor system, the experimental setup should be further optimized. The application of this sensor system will be particularly expected in blood glucose monitoring systems. For application in disposable type sensors, experimental conditions should be optimized considering its dry chemistry, where the component is being re-hydrated by a tiny amount of blood during the measurement. In contrast, the application of this sensor

system for continuous glucose monitoring, requiring utilization in the subcutaneous area for at least 72 h, would necessitate a different optimizations. We are currently investigating the application of the cyt b_{562}-based sensor in the continuous glucose monitoring system, focusing on the lifetime of the protein.

5. Conclusion

In this paper, we describe the construction of glucose sensors employing PQQGDH and GOD coupled with cyt b_{562} as electron acceptor. We demonstrated that glucose enzyme sensor systems employing either PQQGDH or GOD could be constructed without a synthetic electron mediator, but instead using a natural election acceptor. Considering that the natural electron transfer protein is relatively harmless compared with conventional artificial electron acceptors, we expect further application of our system for in vivo sensing systems.

We also demonstrated the potential application of a natural electron transfer proteins as electron transfer subunit for the oxidoreductases that do not naturally possess electron transfer subunits or domains. Although an excess amount of cyt b_{562} is essential in the current condition, further protein engineering approaches may eliminate this need.

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